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(54) Gene encoding enzyme having flavin reducing activity and nitroreductase activity.

(57) The present invention succeeds in isolating a gene encoding an enzyme having an FMN reducing activity and a nitroreductase activity derived from luminous bacteria Vibrio fischeri (ATCC 7744), elucidating its primary structure, and producing Escherichia coli which can express the gene in large quantities. That is, the present invention provides a gene encoding an enzyme having the flavin reducing activity and the nitro-reductase activity, an enzyme produced therefrom, a recombinant vector containing the gene, and bacteria containing the recombinant vector.

Fig. 1

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ATG ACL CAK CCL ATH ATH CAK GAK XTY GAJ AAK WGZ TAK ACL QRS AAJ      48
Met Thr His Pro Ile Ile His Asp Leu Glu Asn Arg Tyr Thr Ser Lys
 1               5                  10                  15
AAJ TAK GAK CCL QRS AAJ AAJ GTL QRS CAJ GAJ GAK XTY GCL GTL XTY      96
Lys Tyr Asp Pro Ser Lys Lys Val Ser Gln Glu Asp Leu Ala Val Leu
            20                  25                  30
XTY GAJ GCL XTY WGZ XTY QRS GCL QRS QRS ATH AAK QRS CAJ CCL TGG     144
Leu Glu Ala Leu Arg Leu Ser Ala Ser Ser Ile Asn Ser Gln Pro Trp
        35                  40                  45
AAJ TTK ATH GTL ATH GAJ QRS GAK GCA GCL AAJ CAJ GGL ATG CAK GAK     192
Lys Phe Ile Val Ile Glu Ser Asp Ala Ala Lys Gln Gly Met His Asp
    50                  55                  60
QRS TTK GCL AAK ATG CAK CAJ TTK AAK CAJ CCL CAK ATH AAJ GCL TGK     240
Ser Phe Ala Asn Met His Gln Phe Asn Gln Pro His Ile Lys Ala Cys
65                  70                  75                  80
QRS CAK GTG ATH XTY TTK GCL AAK AAJ XTY QRS TAK ACL WGZ GAK GAK     288
Ser His Val Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Asp
                85                  90                  95
TAK GAK GTC GTL XTY QRS AAJ GCL GTL GCL GAK AAJ WGZ ATH ACL GAJ     336
Tyr Asp Val Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu
            100                 105                 110
GAJ CAJ AAJ GAJ GCL GCL TTK GCL QRS TTK AAJ TTK GTL CAJ TTG AAK     384
Glu Gln Lys Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn
        115                 120                 125
TGK GAK GAJ AAK GGL CAJ CAK AAJ GCL TGG ACL AAJ CCL CAJ GCL TAK     432
Cys Asp Glu Asn Gly Glu His Lys Ala Trp Thr Lys Pro Gln Ala Tyr
    130                 135                 140
XTY GCL XTY GGL AAK GCL XTY CAK ACL XTY GCL WGZ XTY AAK ATH GAK     480
Leu Ala Leu Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Asp
145                 150                 155                 160
QRS ACL ACL ATC GAJ GGL ATH GAK CCL GAJ XTY TTG QRS GAJ ATH TTK     528
Ser Thr Thr Met Glu Gly Ile Asp Pro Glu Leu Leu Ser Glu Ile Phe
                160                 165                 170                 175
GCL GAK GAJ XTY AAJ GCL TAK GAJ TGK CAK GTL GCL XTY GCL ATH GGL     576
Ala Asp Glu Leu Lys Gly Tyr Glu Cys His Val Ala Leu Ala Ile Gly
            180                 185                 190
TAK CAK CAK CCL QRS GAJ GAK TAK AAK GCL QRS TTG CCL AAJ QRS WGZ     624
Tyr His His Pro Ser Glu Asp Tyr Asn Ala Ser Leu Pro Lys Ser Arg
        195                 200                 205
AAJ GCL TTK GAJ GCL GTL ATH ACL ATH XTY TJJ                         657
Lys Ala Phe Glu Ala Val Ile Thr Ile Leu ***
    210                 215
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TITLE OF THE INVENTION

Gene Encoding Enzyme Having Flavin Reducing Activity and Nitroreductase Activity

BACKGROUND OF THE INVENTION

(i) Field of the Invention

The present invention relates to a gene encoding an enzyme having a flavin reducing activity and a nitroreductase activity, the enzyme produced therefrom, a recombinant vector containing the gene and bacteria containing the recombinant vector.

(ii) Description of the Related Art

A bacterial luciferase derived from luminous bacteria is used to produce oxidized flavin adenine mononucleotide (hereinafter referred to as "oxidized FMN") and a long-chain carboxylic acid in the presence of a long-chain aliphatic aldehyde, oxygen and a reduced flavin adenine mononucleotide (hereinafter referred to as "$FMNH_2$") as a luminescent substrate, and in this case, the bacterial luciferase catalyzes a reaction in which blue light is emitted. $FMNH_2$ which is a substrate can be obtained from a reduced nicotinamide adenine dinucleotide:flavin mononucleotide (NADH:FMN) reductase and a reduced nicotinamide adenine dinucleotide phosphate:flavin mononucleotide (NADPH:FMN) reductase, and the long-chain aldehyde can be obtained from a fatty acid reductase complex.

In recent years, Spyrou et al. have isolated a flavin reductase gene from Escherichia coli and elucidated its primary structure, which is disclosed in Spyrou G., Haggard-Ljungquist E., Krook M., Jornvall H., Nilsson E. and Reichard P., J. Bacteriol, 173, p. 3673-3679 (1991).

Around us, there are many substances (mutagens) which damage chromosomal DNA, and during our lives we are exposed to these substances. Nitroarenes are members of one group of environmental mutagens, and they are contained in the exhaust gas of automobiles, the smoke of incinerators, the atmosphere of cities, the bottoms of rivers, the air in rooms where stoves are lighted, and the burnt portions of grilled chickens. Of nitroarenes having mutability and carcinogenicity, 2-nitrofluorene is well known.

A nitroarene itself does not react directly with DNA to damage the same, but a metabolite of the nitroarene gives rise to a mutation in DNA to damage the DNA. For example, it can be presumed that nitrofluorene is reduced to an N-hydroxy form in the cell of a microorganism by a nitroreductase and then activated by an o-acetyl transferase, to thus finally produce nitrenium ions which attack the DNA. Therefore, it can be considered that the reaction of the nitroreductase with 2-nitrofluorene is a rate determining step in the mutagenesis of DNA by 2-nitrofluorene.

Watanabe et al. have isolated a nitroreductase gene from Salmonella, which is disclosed in Watanabe M., Ishidate M, Jr and Nohmi T., Mutation Research, p. 216 211-220 (1989). Furthermore, its primary structure has been elucidated in Watanabe M., Ishidate M, Jr and Nohmi T., Nucleic Acid Research, 18, p. 1059 (1990).

As understood from the foregoing, the FMN reductase is essential to utilize the luminescent reaction of bacterial luciferase to the utmost. Therefore, the isolation of the FMN reductase gene permits preparing the enzyme in large quantities, and thus, an important object is the isolation of the gene encoding this enzyme.

Furthermore, the nitroreductase gene is useful to improve the detection sensitivity of the above-mentioned mutagen or carcinogen.

However, with regard to the isolation of the FMN reductase gene of luminous bacteria and the nitroreductase gene as well as the expression of them in Escherichia coli, no report has been made so far.

SUMMARY OF THE INVENTION

In view of the above-mentioned technical situation, an object of the present invention is to provide a gene encoding an enzyme having an FMN reducing activity of luminous bacteria and a nitroreductase activity and the enzyme therefor. Another object of the present invention is to provide a recombinant vector containing this gene and bacteria containing the recombinant vector.

As a result of intensive research, the present inventors have succeeded in isolating a gene encoding an enzyme having the FMN reducing activity and the nitroreductase activity from the luminous bacteria Vibrio fischeri (ATCC 7744), and in elucidating its primary structure. In addition, th y have succeeded in cultivating Escherichia coli transformed with a vector containing the gene which can express the protein in large quantities. As a result, the present inv ntion has now b n completed.

The present invention has the following parts (1) to (8).

(1) A gene containing a nucleotide sequence shown in Fig. 1 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

(2) A gen containing a nucleotide sequence shown in Fig. 2 and encoding an enzyme having th flavin reducing activity and th nitroreductase activity describ d in the previous paragraph (1).

(3) A gene containing a nucleotide sequence shown in Fig. 3 and encoding an nzyme having a flavin reducing activity and a nitroreductase activity.

(4) An enzyme containing an amino acid sequence shown in Fig. 4 and having a flavin reducing activity and a nitroreductase activity.

(5) A recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

(6) The recombinant vector described in the previous paragraph (5) in which the gene having the nucleotide sequence shown in Fig. 1 is inserted into a plasmid vector.

(7) Bacteria containing a recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

(8) A method for preparing an enzyme containing an amino acid sequence shown in Fig. 4 which compris s the step of cultivating bacteria transformed with a recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a nucleotide sequence of a gene encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

Sequence length: 657
Sequence type: Nucleic acid
Strandedness: 1
Topology: Linear
Molecular type: Genomic DNA
Feature of sequence description:
Feature key defined in Gene Bank Authorin
Reference Manual Release 1.1 (hereinafter referred to simply as "feature key"): CDS
Procedure for determining the feature:
Prediction from an amino acid sequence of Fig. 4 based on genetic code table.

(On the left and right sides of each triplet, a 5' terminal and a 3' terminal are present, respectively. This triplet represents a purine base (Pu) and a pyrimidine base (Py) constituting a nucleotide sequence.

A: adenine,
G: guanine,
C: cytosine,
J: A or G,
K: T or C,
L: A, T, C or G,
M: A, C or T,
T: thymine,
X: when Y is A or G, X is T or C, or when Y is C or T, X is C,
Y: when X is C, Y is A, G, C or T, or when X is T, Y is A or G,
W: when Z is C or T, W is C or A, or when Z is C or T, W is C,
Z: when W is G, Z is A, G, C or T, or when W is A, W is A or G,
QR: when S is A, G, C or T, QR is TC, and *** represents TAA, TAG or TGA.).

Under each triplet codon of the nucleotide sequence, the amino acid encoded thereby is represented.

Fig. 2 shows a typical nucleotide sequence of the gene encoding the enzyme having a flavin reducing activity and a nitroreductase activity.

Sequence length: 657
Sequence type: Nucleic acid
Strandedness: 1
Topology: Linear
Molecular type: Genomic DNA
Original source:
Organism: Vibrio fischeri
Strain: ATCC 7744

iSDOCID: <EP 0547876A1>

Feature of sequ nce description:
Feature k y d fined in Gene Bank Authorin Referenc Manual R lease 1.1: CDS
Procedure for determining th f ature:
Experimental procedure.

Fig. 3 shows a nucleotide sequence of a gene encoding an enzyme having a flavin reducing activity and a nitroreductase activity.
Sequence length: 929
Sequence type: Nucleic acid
Strandedness: 1
Topology: Linear
Molecular type: Genomic DNA
Original source:
Organism: Vibrio fischeri
Strain: ATCC 7744
Feature of sequence description:
Feature key: CDS
Site having the feature: 109-762
Procedure for determining the feature:
Experimental procedure.

Fig. 4 shows an amino acid sequence of an enzyme having a flavin reducing activity and a nitroreductase activity.
Sequence length: 218
Sequence type: Amino acid
Molecular type: Protein.

Fig. 5 shows an N-terminal amino acid sequence of an NAD(P)H:FMN reductase and synthetic oligonucleotide probes (FR1 and FR2).

Fig. 6 shows a restriction map of the gene of the present invention and a sequencing strategy. Arrows denote directions for the determination of the nucleotide sequences. The portion indicated by a box corresponds to the gene.

Fig. 7 shows a process of constructing a recombinant vector (an expression vector pFR7) containing a gene encoding an enzyme having an FNM reducing activity and a nitroreductase activity of luminous bacteria according to the present invention.

Fig. 8 shows the confirmation of the expressed protein by SDS-polyacrylamide gel electrophoresis. Lane 1 is a pUC8/D1210 strain, lane 2 is a pFR7/D1210 strain, lane 3 is a pFR5/D1210 strain, and lane 4 is a Boehringer Mannheim NAD(P)H:FMN reductase.

The symbols used in the drawings have the following meanings.

lacP	lactose promoter
Amp^r	Ampicillin resistant gene
pUC8	plasmid vector
pFR3	recombinant vector
pFR5	recombinant vector
pFR7	recombinant vector (expression vector)
Sau3AI	four bases (GATC) recognizing restriction enzyme
HincII	six bases (GTPyPuAC) recognizing restriction enzyme
SmaI	six bases (CCCGGG) recognizing restriction enzyme
StuI	six bases (AGGCCT) recognizing restriction enzyme
EcoRI	six bases (GAATTC) recognizing restriction enzyme
HindIII	six bases (AAGCTT) recognizing restriction enzyme
AccI	six bases ($GT^{AT}_{CG}AC$) recognizing restriction enzyme
NspV	six bases (TTCGAA) recognizing restriction enzyme
EcoRV	six bases (GATATC) recognizing restriction enzyme.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The gene of the present invention is characterized by containing a nucleotide sequence having a sequence l ngth of 657 bases as shown in Fig. 1. The nucleotide sequence in Fig.1 can be predicted from an amino acid s quence shown in Fig. 4 as mentioned later.

A preferable sequence contains a nucleotide sequence as shown in Fig. 2.

A typical nucleotide sequence is a DNA having a sequence length of 929 bases as shown in Fig. 3.

The basic nucleotide sequence of the present invention is derived from Genomic DNA isolated from luminous bacteria Vibrio fischeri (ATCC 7744). This sequence is characterized by encoding a protein having a molecular weight of 24562 and comprising 218 amino acids, corresponding to nucleotides numbered 109 to 762.

The gene of the present invention encodes a protein having a flavin reducing activity and a nitroreductase activity, for example, an FMN reducing activity and a nitrofurazone reducing activity.

An enzyme of the present invention is a protein having an amino acid sequence shown in Fig. 4 which can be predicted from the nucleotide sequence in Fig. 3. This protein comprises 218 amino acids and has a molecular weight of 24562 and the two activities of luminous bacteria, i.e., the flavin reducing activity and the nitroreductase activity.

A recombinant vector of the present invention contains a DNA whose nucleotide sequence is shown in Fig. 1. That is, the recombinant vector of the present invention contains a nucleotide sequence which is the same or is functionally equal to the DNA having the nucleotide sequence shown in Fig. 3. A "functionally equal nucleotide sequence" means any DNA fragment which can be used in accordance with a substantially similar method to the present invention so as to obtain the substantially identical results, i.e. the production of an enzyme having the FMN reducing activity and the nitroreductase activity of luminous bacteria in a suitable host.

That is, the "functionally equal nucleotide sequence" means any DNA fragment which can encode a protein having the same amino acid sequence, even if the nucleotide sequence is different, or a DNA fragment which can code a protein having the FMN reducing activity and the nitroreductase activity, even if there is a slight difference in the amino acid sequence attributed to a slight difference in the nucleotide sequence. Typical examples are the nucleotide sequence of Fig. 3 and the nucleotide sequence of Fig. 1 into which a site-specific mutation may be introduced.

The nucleotide sequence in Fig. 1 will be described as follows:

Recently developed techniques make it possible to genetically endow a suitable microorganism with the ability to synthesize a protein or peptide normally made by another organism. The technique makes use of a fundamental relationship which exists in all living organisms between the genetic material, usually DNA, and the proteins synthesized by the organism. This relationship is such that the amino acid sequence of the protein is reflected in the nucleotide sequence of the DNA. There are one or more trinucleotide sequence groups specifically related to each of the twenty amino acids most commonly occurring in proteins. The specific relationship between each given trinucleotide sequence and its corresponding amino acid constitutes the genetic code. The genetic code is believed to be the same or similar for all living organisms. As a consequence, the amino acid sequence of every protein or peptide is reflected by a corresponding nucleotide sequence, according to a well understood relationship. Furthermore, this sequence of nucleotides can, in principle, be translated by any living organism.

The trinucleotides, termed codons, are presented as DNA trinucleotides, as they exist in the genetic material of a living organism. Expression of these codons in protein synthesis requires intermediate formation of messenger RNA (mRNA). The mRNA codons have the same sequences as the DNA codons, except that uracil is found in place of thymine. Complementary trinucleotide DNA sequences having opposite strand polarity are functionally equivalent to the codons, as is understood in the art. An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed. Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid sequence in all organisms, although certain strains may translate some sequences more efficiently than they do others. Occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such methylations do not affect the coding relationship in any way.

The typical example is a plasmid vector into which the DNA fragment having the nucleotide sequence is introduced. As this kind of vector, there can be used pUC [C. Yanisch-Perron, J. Vieira and J. Messing, Gene, 33, p. 110-115 (1985)] and pIN III [Y. Masui, J. Coleman, M. Inouye, Experimental Manipulation of Gene Expression (ed. M. Inouye), Academic Press, p. 15 (1983)].

Fig. 7 shows a construction process of this recombinant vector (the expression vector).

That is, a vector pFR3 having a reductase gene is cleaved with restriction enzymes HincII and StuI to obtain a fragment including a coding region, and this fragment is then inserted into an SmaI site of a pUC8 plasmid DNA [Hanna Z., Fregeau C., Prefontaine G. and Brousseau R., Gene, p. 30247 (1984)] to construct a recombinant vector pFR5. Furthermore, this vector pFR5 is cleaved with a restriction enzyme EcoRI and then subjected to a Klenow treatment in the presence of dNTP. Afterward, the vector is recirculized using a T4 DNA ligase to construct a recombinant vector pFR7 (an expression vector). For the orientation of the thus constructed prod-

uct, a restriction enzyme cleavage site is shown in an ampicillin resistant gene (Amp^r).

Bacteria of the present invention contain a recombinant vector DNA having the nucleotide sequence shown in Fig. 1. The bacteria of the present invention are characterized by producing a protein having the flavin reducing activity and the nitroreductase activity.

A method for preparing the enzyme of the present invention comprises the steps of cultivating bacteria modified with a recombinant vector (an expression vector) containing a DNA whose nucleotide sequence is shown in Fig. 3, and then producing a protein containing an amino acid sequence shown in Fig. 4. Examples of the bacteria include Escherichia coli and Bacillus subtilis, and examples of a culture medium to be used include an LB culture medium and a YT culture medium.

A gene of the present invention is that which has been isolated for the first time encoding an enzyme having an FMN reducing activity and a nitroreductase activity. This gene can be used to produce a highly sensitive strain of bacteria to a mutagen or a carcinogen by the use of a suitable host such as Escherichia coli. Additionally, from this Escherichia coli, a reductase protein can also be prepared in large quantities.

By inserting this expression vector into a suitable host such as Escherichia coli, organisms or bacteria can be produced which express an enzyme having the FMN reducing activity and the nitroreductase activity of luminous bacteria. Furthermore, the reductase can also be prepared in large quantities by extraction from the organisms into which the gene is introduced. The organisms or microorganisms into which the gene is introduced have a high sensitivity to a mutagen or a carcinogen owing to the above-mentioned function, and thus they are useful as an indicator for detecting the mutagen or the carcinogen.

The reductase amplifies a luminous reaction of bacterial luciferase owing to the above-mentioned function. Thus, the reductase can be applied to many measuring methods and it is useful, for example, as a diagnosis drug or an inspection drug.

EXAMPLES

Now, the isolation and identification of a gene which is important to the present invention will be described in reference to examples.

Example 1

[Identification of NAD(P)H:FMN reductase and determination of N-terminal amino acid sequence]

An NAD(P)H:FMN reductase sample (available from Boehringer Mannheim) was introduced into a "Sparose 12" gel filtration column (made by Pharmacya Co., Ltd.) to fractionate the sample. For each fraction, NADH and NADPH:FMN reducing activities were measured by a procedure described in Jablonski E. and DeLuca M., Biochemistry, 16, p. 2932 (1977), and analysis was then made in accordance with a procedure described in Laemmli, U.K. Nature, 277, p. 680 (1970) by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

As a result, it was clarified that the FMN reducing activity is directly proportional to the amount of a protein of 26 kDa (which is denoted by an arrow in Fig. 8).

After an SDS-PAGE analysis of this protein, it was transferred into a nylon membrane, and its amino acid sequence was determined in a usual manner by the use of a protein sequencer [made by Applied Biosystems Inc. (ABI)]. The results are set forth in Fig. 5. From these results, an N-terminal amino acid sequence having sequence numbers of 1 to 24 was confirmed.

Example 2

[Preparation of luminescent bacteria genomic library]

A photobacterium medium containing luminescent bacteria Vibrio fischeri (ATCC7744) was shaken at 26°C overnight to cultivate the bacteria. The bacteria were collected by means of centrifugal separation at 10000 rpm, and the resultant cell pellets were then dispersed in a Tris-HCl·EDTA buffer solution (hereinafter referred to as "a TE buffer"). After a lysozyme treatment at 37°C for 1 hour, sodium dodecyl sulfate (hereinafter abbreviated to "SDS") was added, followed by a proteinase K treatment at 50°C for 3 hours. Afterward, a phenol treatment was carried out three times, followed by ethanol precipitation. After drying, the dried material was dissolved in the TE buffer, and then subjected to the proteinase K treatment again. Afterward, the three cycles of the phenol treatment and then the ethanol precipitation were carried out to recover the genomic DNA. 10 units of a restriction enzyme Sau3AI were reacted with 50 μm of this genomic DNA at 37°C. Some parts of the reaction

mixtur were taken out at reaction times of 5, 10, 20, 30, 45, 60, 90 and 120 minutes, and afterward, EDTA (ethylen diaminetetraacetic acid) was added to the reaction system to bring the reaction to an nd. Each part of the DNA was subj ct d to agarose gel electrophoresis to confirm the degree of partial decomposition of the genomic DNA. The reaction solutions at the respective times were combined into one, followed by the ethanol precipitati n, to recover the DNA. Next, this DNA was dissolv d in a small amount f th TE buffer, and then subj cted t agarose gel lectroph resis t recover a fraction of 4 to 6 Kb by the use of a DE81 pap r. The DNA fraction of 4 to 6 Kb was dissolved out of the DE81 paper with 1 M NaCl, subjected to the phenol treatment three times, and then precipitated with ethanol. The sample was dissolved in the TE buffer so as to be about 200 ng/μl. Afterward, the DNA fraction of 4 to 6 Kb was reacted with a pUC18 plasmid DNA (a plasmid vector), which was previously cleaved with a restriction enzyme BamH I and then treated with an alkaline phosphatase (an enzyme for catalyzing dephosphorization at the 5' terminal of the DNA), at 16°C overnight in the presence of a T4 DNA ligase (an enzyme for ligating DNA chains to each other or ligating the DNA and the 3'OH of an RNA or the 5'P terminal by a phosphodiester bond), whereby the DNA fraction was ligated to the plasmid. The resultant ligation reaction solution was transferred to JM109 Escherichia coli so as to perform transformation, and the thus obtained transductant represented a gene library.

[Preparation of synthetic oligonucleotide probe]

On the basis of the information of an amino acid sequence shown in Fig. 5, two probes of an oligonucleotide probe (FR-1) and an oligonucleotide probe (FR-2) were synthesized by means of a DNA synthesizer (made by ABI). Each synthetic probe was purified by the use of an OPC cartridge (made by ABI).

[Cloning of NAD(P)H:FMN reductase gene and analysis of its structure]

The gene library of Example 2 was screened in accordance with a colony hybridization method by the use of the FR-1 probe and the FR-2 probe. The FR-1 probe and the FR-2 probe were labelled at the 5' terminal with [γ-^{32}P]ATP and then used as labelled probes. After the titer of the gene library was measured, this g ne library was scattered on a nitrocellulose filter so as to be 200 colonies per plate. Cultivation was made at 37°C overnight, and two replicas were taken per filter. Each pair of two replicas was cultivated at 37°C and then used for hybridization. The filter was air-dried and then irradiated with ultraviolet rays (UV) to fix the DNA. Afterward, the filter was put in a hybridization solution {20 ml of a 6xSET buffer [20xSET buffer:3 M of NaCl, 0.6 M of Tris-HCl (pH 8.0) and 0.04 M of EDTA], a 10xDehhardt's solution [(a solution containing 0.2% of each of serum albumin, polyvinylpyrrolidone and Ficoll), a 0.1% SDS and a salmon sperm DNA (thermally denatured, 50 μm/ml)], and it was then maintained at 68°C for 1 hour. Furthermore, the solution was replaced with a new one and then maintained for 1 hour, and a ^{32}P-levelling probe was added, followed by hybridization at room temperature overnight. The solution was thrown away, and the filter was then washed with the 6xSET buffer, followed by shaking at 37°C for 20 minutes. After this operation was repeated twice, the filter was air-dried and then subjected to autoradiography. The filter was superposed upon a developed X-ray film, and the position of an ink marker was photographed on the film. Identification was made by aligning signals which were coincident with each other on the two films of the probe FR-1 and the probe FR-2 made from the one plate, and thus, five identified colonies (clones) were obtained.

[Preparation of recombinant vector]

For these five clones, a restriction analysis was carried out (Fig. 6), and as a result, it was apparent that three of these five clones were the same clones, which meant that three kinds of positive clones were prepared. Above all, a recombinant vector pFR3 (Fig. 7) having the smallest inserted DNA was used for the subsequent analysis.

[Structure determination of the gene and determination of amino acid sequence]

A Southern blotting analysis was made by the use of the FR-1 probe, and the region of an FMN reductase gene was determined in accordance with a dideoxynucleotide-enzyme method [Hattori M. and Sakaki Y., Anal, Biochem., 152, p. 232 (1986)], wh reby a primary structure shown in Fig. 3 was elucidated. As a result, it was understood that the FMN reductas gene encoded a polypeptide of 24562 Da comprising 218 amino acids shown in Fig. 4, and this gene was about 30% homologous with a nitroreductase gene of Salmonella [Watanab M., Ishidate M. Jr and Nohmi T., Nucleic Acid Res., 18, p. 1059 (1990)].

Example 3

[Recombinant vector of NAD(P)H:FMN reductase gen , and construction of expression vector] (Fig. 7)

A recombinant v ctor pFR3 plasmid DNA was cleaved with a restriction enzyme Hinc II/Stu I and then treated at -80°C for 10 minutes. The thus treated DNA was then subjected to agarose gel electrophoresis to separate and recover a DNA fragment of about 1 Kb by the us of a DE81 paper. The DNA was diss lved out of the DE81 paper with 1 M NaCl, subjected to the phenol treatment three times, and then precipitated with ethanol. Next, the sample was dissolved in the TE buffer so as to be about 200 ng/μl. The above-mentioned DNA was reacted, at 16°C overnight in the presence of a T4DNA ligase, with a pUC8 plasmid DNA (a plasmid vector) which was previously cleaved with a restriction enzyme Sma I and then treated with an alkaline phosphatase, whereby the DNA was ligated to the plasmid. The resultant ligation reaction solution was transferred to JM109 Escherichia coli to perform transformation, and the Escherichia coli was selected and then cultivated overnight in a culture medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) to form a white colony. This white colony was a transductant containing the plasmid into which the heterologous DNA was inserted.

A plasmid DNA was prepared from these transductants, and a restriction analysis was then carried out to obtain a strain containing a transformed vector pFR5. A plasmid DNA of the recombinant vector pFR5 was prepared, cleaved with EcoR I, subjected to a Klenow treatment, ligated with a T4DNA ligase, and then was transferred to D1210 Escherichia coli to perform transformation. Of the transductants, one in which an EcoR I cleavage site disappeared was selected. This was a recombinant vector (an expression vector) pFR7.

The recombinant vector pFR5 was constructed so as to express a peptide derived from a N-terminal β-galactosidase gene (lacZ) and a fused protein of the FMN reductase enzyme. The expression vector pFR7 was constructed so as to express lacZ and frameshift FMN reductase singly.

Example 4

[Preparation of Escherichia coli incorporated with NAD(P)H:FMN reductase gene]

Expression vectors pFR5 and pFR7 and a pUC8 plasmid DNA were transferred to D1210 Escherichia coli to perform transformation.

[Preparation of enzyme]

These transductants were incubated overnight, and 0.25 ml of the resultant incubation solution was transferred to an LB liquid (10 ml) culture medium containing ampicillin. After the culture medium was shaken at 37°C for 2 hours to cultivate the transductants, isopropyl-β-D(-)-thiolactopyranoside (hereinafter abbreviated to "IPTG") was added thereto so that a final concentration might be 1 mM, and the transductants were further cultivated for 3 hours. For the bacteria, an SDS-PAGE analysis was carried out to confirm the expression of a protein (this protein corresponds to the enzyme of the present invention).

The results are set forth in Fig. 4, but in the cases of the recombinant vectors pFR5 and pFR7, new bands appeared at 26 kDa which was the same size as in a commercial crude enzyme sample. In addition, in the case of the recombinant vector pFR5, a band appeared even at 29 kDa, and this vector was considered to be derived from a fused protein with lac Z.

1.5 ml of the incubation solution which was subjected to an IPTG induction treatment was centrifugally separated at 10000 rpm to remove a supernatant. The bacteria were dispersed in 0.5 ml of a 50 mM potassium phosphate·1 mM dithiothreitol buffer, and then sonically disrupted by ultrasound. Centrifugal separation was further carried out at 4°C for 30 minutes at 12000 rpm, and the resultant supernatant was a cell extract.

For this cell extract, the following enzyme reducing activity was measured. The results are set forth in Tables 1, 2 and 3.

(1) Flavin reducing activity: This was measured in accordance with a procedure described in Jablonski E and Deluca M., Biochemistry, 16, p. 2932 (1977).

(2) Iron reducing activity: This was measured in accordance with a procedure described in Fontecave M., Eliasson R. and Reichard P., J. Biol. Chem., 262, p. 12325-12331 (1987).

(3) Nitroreductase activity: This was measured in accordance with a procedure described in Watanabe M., Ishidate M. Jr. and Nohmi T., Mutation Research, 216, p. 211-220 (1989).

Protein amounts in the respective tables were determined in accordance with a Bradford method by th us of a protein assary kit made by Bio-RAD [Bradford M. M., Anal. Biochem., 72, p. 248-254 (1976)].

Table 1

	Strain (IPTG)	Flavin Reducing Activity (nmol/min/mg protein) FMN (+)	FMN (-)	FAD (+)	FAD (-)	Riboflavin (+)	Riboflavin (-)
NADH	pFR5/D1210	6330	430	3980	240	2120	80
	pFR7/D1210	11810	660	7410	340	2700	50
	pUC13/D1210	20	30	50	40	10	0
	D1210	40	30	40	10	10	0
NADPH	pFR5/D1210	2300	190	1680	70	660	10
	pFR7/D1210	4430	230	2840	140	870	110
	pUC13/D1210	50	30	0	10	0	50
	D1210	20	50	10	30	10	0

Table 2

	Strain (IPTG)	Iron Reductase Activity (nmol/min/mg protein) FMN (+)	FMN (-)	FAD (+)	FAD (-)	Riboflavin (+)	Riboflavin (-)
NADH	pFR5/D1210	44.9	4.5	39.4	3.6	30.1	2.9
	pFR7/D1210	68.3	6.6	59.5	5.4	42.8	4.2
	pUC13/D1210	0.4	0.1	1.6	1.7	0.1	0.0
	D1210	0.4	0.2	1.3	1.8	0.0	0.0
NADPH	pFR5/D1210	12.6	1.5	11.8	0.4	9.4	0.3
	pFR7/D1210	25.8	2.5	23.6	1.3	14.0	0.5
	pUC13/D1210	0.6	0.4	0.1	0.0	0.2	0.0
	D1210	0.6	0.3	0.1	0.0	0.4	0.0

Tabl 3

	Strain (IPTG)	Nitroreductase Activity (nmol/min/mg protein) Nitrofurazone	
		(+)	(-)
NADPH	pFR5/D1210	24.8	10.1
	pFR7/D1210	40.9	10.4
	pUC13/D1210	4.7	5.2
	D1210	6.8	5.5

Comparing the activities in these tables, the activities of pFR5 and pFR7 are about 1 to 3 orders higher than those of pUC13 which is a negative control. This gene could therefore be identified as a gene encoding an enzyme protein having a flavin reducing activity and a nitroreductase activity.

Claims

1. An isolated and purified gene containing a nucleotide sequence shown in Fig. 1 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

2. An isolated and purified gene containing a nucleotide sequence shown in Fig. 2 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

3. An isolated and purified gene containing a nucleotide sequence shown in Fig. 3 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

4. An enzyme containing an amino acid sequence shown in Fig. 4 and having a flavin reducing activity and a nitroreductase activity.

5. A recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

6. The recombinant vector according to Claim 5 wherein the nucleotide sequence shown in Fig. 1 is insert d into a plasmid vector.

7. A bacterial host containing a recombinant vector which vector contains a DNA whose nucleotide sequence is shown in Fig. 1.

8. A method for preparing an enzyme containing an amino acid sequence shown in Fig. 4 which comprises a step of cultivating bacteria modified with a recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

Fig. 1

```
ATG ACL CAK CCL ATM ATM CAK GAK XTY GAJ AAK WGZ TAK ACL QRS AAJ        48
Met Thr His Pro Ile Ile His Asp Leu Glu Asn Arg Tyr Thr Ser Lys
 1               5                  10                  15
AAJ TAK GAK CCL QRS AAJ AAJ GTL QRS CAJ GAJ GAK XTY GCL GTL XTY        96
Lys Tyr Asp Pro Ser Lys Lys Val Ser Gln Glu Asp Leu Ala Val Leu
            20                  25                  30
XTY GAJ GCL XTY WGZ XTY QRS GCL QRS QRS ATM AAK QRS CAJ CCL TGG       144
Leu Glu Ala Leu Arg Leu Ser Ala Ser Ser Ile Asn Ser Gln Pro Trp
        35                  40                  45
AAJ TTK ATM GTL ATM GAJ QRS GAK GCA GCL AAJ CAJ GGL ATG CAK GAK       192
Lys Phe Ile Val Ile Glu Ser Asp Ala Ala Lys Gln Gly Met His Asp
    50                  55                  60
QRS TTK GCL AAK ATG CAK CAJ TTK AAK CAJ CCL CAK ATM AAJ GCL TGK       240
Ser Phe Ala Asn Met His Gln Phe Asn Gln Pro His Ile Lys Ala Cys
65                  70                  75                  80
QRS CAK GTG ATM XTY TTK GCL AAK AAJ XTY QRS TAK ACL WGZ GAK GAK       288
Ser His Val Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Asp
                85                  90                  95
TAK GAK GTG GTL XTY QRS AAJ GCL GTL GCL GAK AAJ WGZ ATM ACL GAJ       336
Tyr Asp Val Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu
            100                 105                 110
GAJ CAJ AAJ GAJ GCL GCL TTK GCL QRS TTK AAJ TTK GTL CAJ TTG AAK       384
Glu Gln Lys Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn
        115                 120                 125
TGK GAK GAJ AAK GGL GAJ CAK AAJ GCL TGG ACL AAJ CCL CAJ GCL TAK       432
Cys Asp Glu Asn Gly Glu His Lys Ala Trp Thr Lys Pro Gln Ala Tyr
    130                 135                 140
XTY GCL XTY GGL AAK GCL XTY CAK ACL XTY GCL WGZ XTY AAK ATM GAK       480
Leu Ala Leu Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Asp
145                 150                 155                 160
QRS ACL ACL ATG GAJ GGL ATM GAK CCL GAJ XTY TTG QRS GAJ ATM TTK       528
Ser Thr Thr Met Glu Gly Ile Asp Pro Glu Leu Leu Ser Glu Ile Phe
                160                 170                 175
GCL GAK GAJ XTY AAJ GGL TAK GAJ TGK CAK GTL GCL XTY GCL ATM GGL       576
Ala Asp Glu Leu Lys Gly Tyr Glu Cys His Val Ala Leu Ala Ile Gly
            180                 185                 190
TAK CAK CAK CCL QRS GAJ GAK TAK AAK GCL QRS TTG CCL AAJ QRS WGZ       624
Tyr His His Pro Ser Glu Asp Tyr Asn Ala Ser Leu Pro Lys Ser Arg
        195                 200                 205
AAJ GCL TTK GAJ GCL GTL ATM ACL ATM XTY TJJ                           657
Lys Ala Phe Glu Ala Val Ile Thr Ile Leu ***
    210                 215
```

Fig. 2

```
ATG ACG CAT CCA ATT ATT CAT GAT CTT GAA AAT CGT TAT ACA TCA AAA      48
Met Thr His Pro Ile Ile His Asp Leu Glu Asn Arg Tyr Thr Ser Lys
 1               5                  10                  15
AAA TAT GAC CCA TCA AAG AAA GTA TCT CAA GAA GAT TTA GCG GTT TTG      96
Lys Tyr Asp Pro Ser Lys Lys Val Ser Gln Glu Asp Leu Ala Val Leu
            20                  25                  30
CTT GAG GCT CTG CGT TTA TCT GCT TCT TCA ATT AAT TCA CAG CCT TGG     144
Leu Glu Ala Leu Arg Leu Ser Ala Ser Ser Ile Asn Ser Gln Pro Trp
        35                  40                  45
AAA TTC ATT GTT ATT GAA TCC GAT GCA GCG AAG CAA GGT ATG CAT GAT     192
Lys Phe Ile Val Ile Glu Ser Asp Ala Ala Lys Gln Gly Met His Asp
    50                  55                  60
TCG TTT GCA AAT ATG CAT CAG TTT AAT CAA CCT CAC ATC AAA GCG TGT     240
Ser Phe Ala Asn Met His Gln Phe Asn Gln Pro His Ile Lys Ala Cys
65                  70                  75                  80
TCT CAT GTG ATT TTA TTT GCA AAT AAG CTT TCG TAT ACA CGA GAT GAT     288
Ser His Val Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Asp
                85                  90                  95
TAT GAT GTG GTT TTA TCT AAA GCG GTT GCT GAC AAG CGT ATT ACT GAA     336
Tyr Asp Val Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu
            100                 105                 110
GAG CAA AAA GAA GCT GCT TTT GCT TCG TTT AAG TTT GTA GAA TTG AAC     384
Glu Gln Lys Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn
        115                 120                 125
TGT GAT GAA AAT GGT GAG CAT AAA GCA TGG ACT AAG CCT CAA GCT TAT     432
Cys Asp Glu Asn Gly Glu His Lys Ala Trp Thr Lys Pro Gln Ala Tyr
    130                 135                 140
TTA GCT CTT GGT AAT GCT CTG CAT ACA TTA GCT AGA CTG AAC ATT GAC     480
Leu Ala Leu Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Asp
145                 150                 155                 160
TCA ACA ACA ATG GAA GGC ATT GAT CCT GAA TTA TTG AGT GAA ATT TTT     528
Ser Thr Thr Met Glu Gly Ile Asp Pro Glu Leu Leu Ser Glu Ile Phe
                160                 170                 175
GCT GAT GAA TTA AAA GGG TAT GAA TGT CAT GTT GCT TTA GCC ATT GGT     576
Ala Asp Glu Leu Lys Gly Tyr Glu Cys His Val Ala Leu Ala Ile Gly
            180                 185                 190
TAT CAT CAT CCA AGC GAA GAT TAT AAT GCC TCT TTG CCT AAG TCT CGT     624
Tyr His His Pro Ser Glu Asp Tyr Asn Ala Ser Leu Pro Lys Ser Arg
        195                 200                 205
AAG GCA TTT GAA GCA GTA ATT ACC ATC CTT TAG                        657
Lys Ala Phe Glu Ala Val Ile Thr Ile Leu ***
    210                 215
```

Fig. 3

```
TGTCACATAT GGCAAATTAA ATATTGAGTA TGCCTTGCTT GTTGACATCA TAAGTTGTGC     60
AGACAAGAAT GTCTGTGGAT TAAAATTTCA CAAGTAAGGT TTATTATT ATG ACG CAT     117
                                                      Met Thr His
                                                       1
CCA ATT ATT CAT GAT CTT GAA AAT CGT TAT ACA TCA AAA AAA TAT GAC       165
Pro Ile Ile His Asp Leu Glu Asn Arg Tyr Thr Ser Lys Lys Tyr Asp
     5                  10                  15
CCA TCA AAG AAA GTA TCT CAA GAA GAT TTA GCG GTT TTG CTT GAG GCT      213
Pro Ser Lys Lys Val Ser Gln Glu Asp Leu Ala Val Leu Leu Glu Ala
 20                  25                  30                  35
CTG CGT TTA TCT GCT TCT TCA ATT AAT TCA CAG CCT TGG AAA TTC ATT       261
Leu Arg Leu Ser Ala Ser Ser Ile Asn Ser Gln Pro Trp Lys Phe Ile
                 40                  45                  50
GTT ATT GAA TCC GAT GCA GCG AAG CAA GGT ATG CAT GAT TCG TTT GCA       309
Val Ile Glu Ser Asp Ala Ala Lys Gln Gly Met His Asp Ser Phe Ala
             55                  60                  65
AAT ATG CAT CAG TTT AAT CAA CCT CAC ATC AAA GCG TGT TCT CAT GTG       357
Asn Met His Gln Phe Asn Gln Pro His Ile Lys Ala Cys Ser His Val
         70                  75                  80
ATT TTA TTT GCA AAT AAG CTT TCG TAT ACA CGA GAT GAT TAT GAT GTG       405
Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Asp Tyr Asp Val
     85                  90                  95
GTT TTA TCT AAA GCG GTT GCT GAC AAG CGT ATT ACT GAA GAG CAA AAA       453
Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu Glu Gln Lys
100                 105                 110                 115
GAA GCT GCT TTT GCT TCG TTT AAG TTT GTA GAA TTG AAC TGT GAT GAA       501
Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn Cys Asp Glu
                120                 125                 130
AAT GGT GAG CAT AAA GCA TGG ACT AAG CCT CAA GCT TAT TTA GCT CTT       549
Asn Gly Glu His Lys Ala Trp Thr Lys Pro Gln Ala Tyr Leu Ala Leu
            135                 140                 145
GGT AAT GCT CTG CAT ACA TTA GCT AGA CTG AAC ATT GAC TCA ACA ACA       597
Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Asp Ser Thr Thr
        150                 155                 160
ATG GAA GGC ATT GAT CCT GAA TTA TTG AGT GAA ATT TTT GCT GAT GAA       645
Met Glu Gly Ile Asp Pro Glu Leu Leu Ser Glu Ile Phe Ala Asp Glu
    160                 170                 175
TTA AAA GGG TAT GAA TGT CAT GTT GCT TTA GCC ATT GGT TAT CAT CAT       693
Leu Lys Gly Tyr Glu Cys His Val Ala Leu Ala Ile Gly Tyr His His
180                 185                 190                 195
CCA AGC GAA GAT TAT AAT GCC TCT TTG CCT AAG TCT CGT AAG GCA TTT       741
Pro Ser Glu Asp Tyr Asn Ala Ser Leu Pro Lys Ser Arg Lys Ala Phe
                200                 205                 210
GAA GCA GTA ATT ACC ATC CTT                                          762
Glu Ala Val Ile Thr Ile Leu
            215
TAGATTCTTA ATGTTTGAGA TGAAGAAAAG CCAGCGATTT AGCTGTGCTT TGTTTGTGCA     822
***
AAAATGTTCC TAATGGCGTA TTACTACGGT AGGAAGTCTA TTTAAAGTTT CTTTTACTCT     882
TTGGTATTAA TTGTCAATTA CGCGGAAATC ATTATCTAAC TAGGCCT                   929
```

Fig. 4

```
Met Thr His Pro Ile Ile His Asp Leu Glu Asn Arg Tyr Thr Ser Lys
 1               5                  10                  15
Lys Tyr Asp Pro Ser Lys Lys Val Ser Gln Glu Asp Leu Ala Val Leu
            20                  25                  30
Leu Glu Ala Leu Arg Leu Ser Ala Ser Ser Ile Asn Ser Gln Pro Trp
        35                  40                  45
Lys Phe Ile Val Ile Glu Ser Asp Ala Ala Lys Gln Gly Met His Asp
    50                  55                  60
Ser Phe Ala Asn Met His Gln Phe Asn Gln Pro His Ile Lys Ala Cys
65                  70                  75                  80
Ser His Val Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Asp
                85                  90                  95
Tyr Asp Val Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu
            100                 105                 110
Glu Gln Lys Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn
        115                 150                 125
Cys Asp Glu Asn Gly Glu His Lys Ala Trp Thr Lys Pro Gln Ala Tyr
    130                 135                 140
Leu Ala Leu Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Asp
145                 150                 155                 160
Ser Thr Thr Met Glu Gly Ile Asp Pro Glu Leu Leu Ser Glu Ile Phe
                165                 170                 175
Ala Asp Glu Leu Lys Gly Tyr Glu Cys His Val Ala Leu Ala Ile Gly
            180                 185                 190
Tyr His His Pro Ser Glu Asp Tyr Asn Ala Ser Leu Pro Lys Ser Arg
        195                 200                 205
Lys Ala Phe Glu Ala Val Ile Thr Ile Leu
    210                 215
```

Fig.5

```
                    5                   10
Met-Thr-His-Pro-Ile-Ile-His-Asp-Leu-Glu-Asn-Arg-Tyr

FR1 : 5'ATG-ACT-CAT-CCT-AT 3'
              C   C   C
              A       A
              G       G

          15                  20
-Thr-Ser-Lys-Lys-Tyr-Asp-Pro-Ser-Lys-Lys-Val- . . .

FR2 : 5'AAA-AAA-TAT-GAT-CC 3'
            G   G   C   C
```

Fig.6

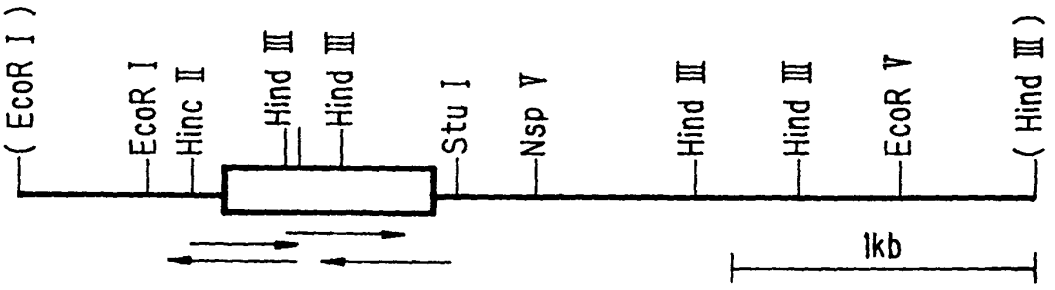


Fig.7

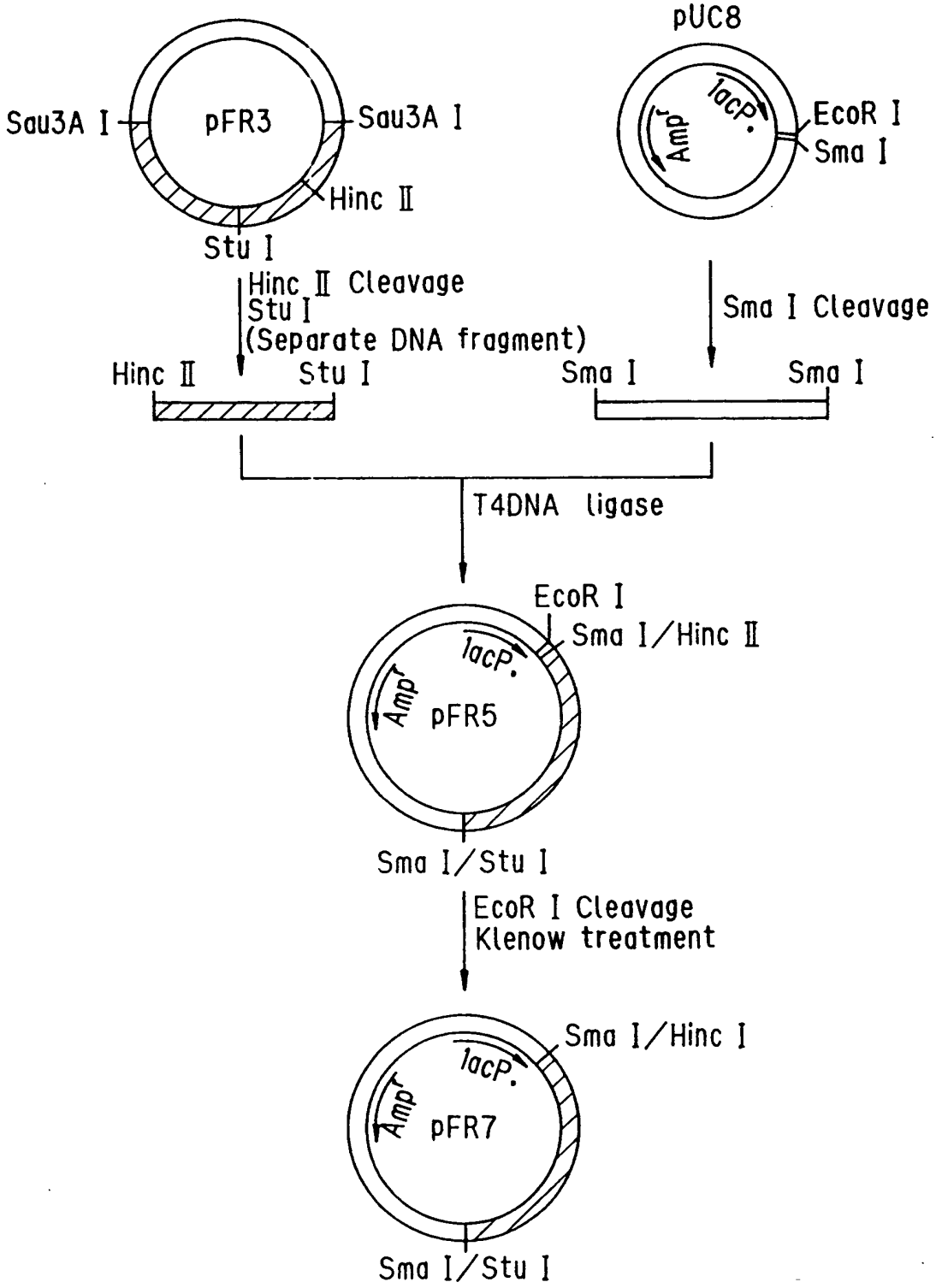
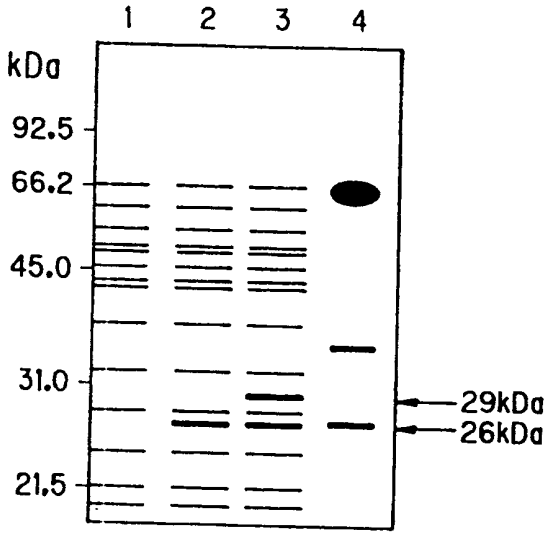


Fig.8



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Application Number

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PAGE1

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	16 MARCH 1993	JULIA P.

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The present search report has been drawn up for all claims

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